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Review

Inorganic pyrophosphate in mitochondrial metabolism

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Contents

This article reviews PP_i metabolism in mitochondria and possible role of PP_i in regulation of different processes in them.

I. Introduction

Inorganic pyrophosphate (PP_i) was for a long time believed to be a byproduct of biosynthetic reactions subject to immediate hydrolysis by inorganic pyrophosphatases (PPase) [1]. But as of the early 1960's, data have been accumulated on the important bioenergetic and regulatory role of this compound. Now, after broad-based investigations, the impression is that in animal tissues PP_i has an important part to play in the regulation of calcium-phosphate metabolism, while deviations of its concentrations from definite values in tissue liquids may indicate a variety of pathologies [2]. In microorganisms specific PP_i-dependent enzymes have been detected, responsible for the phosphorylation of essential intermediates of glycolysis and glucongeogenesis [3,4]. Simultaneously, it was found that the polyfunctional enzyme from liver cells, glucose-6-phosphatase, is also capable of effecting the PP_i-dependent synthesis of glucose-6-phosphate [5–11]. PP_i-dependent enzymes were found to be widespread in plants as well [12–17]. An, finally, in 1966, H. Baltscheffsky and co-workers [18,19] detected light-dependent synthesis of PP_i in the nonsulfur, purple photosynthetic bacterium, *Rhodospirillum rubrum*, and Baltscheffsky's team [20,21] found that this compound may be used at the level of electron

Abbreviations: PP_i, inorganic pyrophosphate; PPase, inorganic pyrophosphatase; F_1, coupling factor 1 (or ATPase); CF_1, coupling factor 1 of chloroplasts; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical potential difference across the inner mitochondrial membrane; $\Delta\psi$, electrical component of $\Delta\mu_{H^+}$; ΔpH, chemical component of $\Delta\mu_{H^+}$.

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transport chain. PP_i biosynthesis, coupled to the respiratory chain, was described for yeast [22], animal [23,24] and plant [25,26] mitochondria, and that coupled to the photosynthetic electron transport chain – for alga and higher plant chloroplasts [27]. A major discovery in the past few years was the PP_i transport across the inner mitochondrial membrane in exchange for ADP via the adenine nucleotide translocator [28–31] and the generation of an electrochemical potential ($\Delta\tilde{\mu}_{H^+}$) across the membrane of microorganisms [32–34], the inner membrane of mitochondria [35] and plant cell membranes [36–38] at the expense of the energy liberated under PP_i hydrolysis.

This cumulative evidence goes to show that PP_i is not only a byproduct of pyrophosphorolysis reactions and not only a compound utilized in the bioenergetics of the primitive forms of life – it is also a macroerg that plays a significant role in the bioenergetic of contemporary higher organisms. It has been found that inorganic pyrophosphatases include membrane enzymes not only capable of hydrolyzing PP_i with the scatter of its phosphoanhydride bond energy but also capable of involvement in energy transformation reactions and of transferring this energy from the chemical to the electric form and vice versa.

II. PP_i sources in mitochondria

Pyrophosphate has been detected in mitochondria by a number of researchers [39–42]. Its content in liver mitochondria is about several μmol/g of the protein and depends on the animals' diet.

One of the sources of PP_i in these organelles may be numerous pyrophosphorolysis reactions, specifically, fatty acid activation reactions:

$$\text{butyrate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{butyryl-CoA} + PP_i + \text{AMP}$$

So far as is known [43,44], butyrate is activated only in mitochondria.

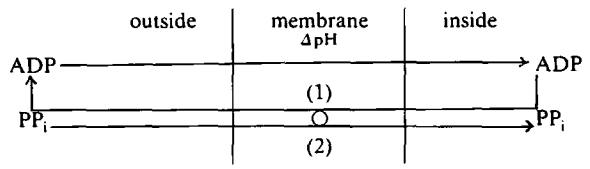
According to the data of Veech and co-workers [11], the intraperitoneal injection of butyrate to rats, fasted for 72 h, caused an increase in the PP_i concentration in liver mitochondria to 20 mM, and this occurred in the presence of soluble and membrane mitochondrial pyrophosphatases [24,45–50]. Such accumulation is possible, on the one hand, in the presence of enzyme inhibitors, e.g., Ca^{2+} ions [45], and as a result of subtle regulation of the activity of the enzyme. That such kind of regulation is possible is testified by the results of our recent experiments which have demonstrated that well-coupled rat liver mitochondria, incubated for 10 min in the presence of ATP and butyrate, can accumulate over 10 μmol of PP_i per g of the mitochondrial protein in the absence of a pyrophosphatase inhibitor as well. Addition of even small amounts of uncouplers resulted in the immediate hydrolysis of accumulated PP_i. This evidence has shown that the level of PP_i in mitochondria may be controlled by a difference in electric potentials across the inner membrane.

Another source of PP_i in mitochondria may be electron-transport-coupled biosynthesis of PP_i with the participation of the coupling membrane pyrophosphatase. As we have already said, such possibility was shown for yeast mitochondria [22], as well as for mitochondria of animal [23] and plant tissues [25,26]. Just as in the case of *R. rubrum* chromatophores, PP_i biosynthesis in mitochondria depended on the work of the electron transport chain; it proceeded parallel to ATP biosynthesis, was inhibited by uncouplers and fluoride at a concentration suppressing the hydrolase activity of mitochondrial pyrophosphatases but exerting little, if any, effect on ATP hydrolysis and synthesis. Oligomycin, the specific inhibitor of mitochondria ATPase, did not inhibit the hydrolysis and synthesis of PP_i. The PP_i accumulation rate by rat liver mitochondria on succinate at 18° C was about 2 μmol, and by rat heart mitochondria 5 μmol/g of the protein per min. Significantly higher figures were reported for plant mitochondria [26]. Submitochondrial particles, depleted of matrix enzymes, including soluble pyrophosphatase, likewise effected electron transport chain-coupled biosynthesis of PP_i under conditions of oxidative phosphorylation on succinate, glutamate + malate or NADH [51]. Evidence for the coupling role of membrane pyrophosphatase from mitochondria was also obtained in experiments on reconstruction of the PP_i synthesis system by using submitochondrial particles washed of pyrophosphatase and the isolated enzyme [24,52].

Beginning with works by Schick and Butler [53], and Batenburg and Van den Bergh [54], the inner membrane of mitochondria was known to be impermeable to PP_i. On the other hand, it was clear that PP_i is capable of penetrating mitochondria. That followed, for example, from the experiments on increasing ATP synthesis by hydrolyzing a PP_i added to the medium [23,52], and also from experimental results of Veech and co-workers [11], who found redistribution of PP_i and adenine nucleotides between the cytosol and mitochondria as butyrate was activated in liver cells.

Finally, in 1980 Asimakis and Aprille [28,29] reported on adenine nucleotide translocator-mediate PP_i transport in mitochondria in exchange for ADP. Subsequently these data were confirmed in other works [30,31,35]. It was shown that inhibitors of adenine nucleotide transport (carboxyatractyloside, bongkrekic acid and palmitoyl-CoA) also suppress PP_i transport into mitochondria in the presence of ADP. The final proof of the involvement of adenine nucleotide translocator in this process was obtained by Krämer [31] in experiments on reconstitution of the system from liposomes and a translocator isolated from beef heart mito-

chondria. The exchange stoichiometry proved to be the same as in the case of ADP–ATP exchange, i.e., 1 mol of PP_i per mol of the nucleotide. Yet it was shown at the same time that mitochondria have a system different from the adenine nucleotide translocator and capable of transporting ADP (ATP) into mitochondria in the presence of phosphate at the expense of the energy caused by the difference of hydrogen ion concentrations (ΔpH) [28,56]. PP_i transfer into mitochondria with the participation of these two transporters may be schematically represented as follows:



A ΔpH-dependent system of transport allows, in the presence of phosphate (its role is yet not clear), accumulation of adenine nucleotides in mitochondria in amounts by an order of magnitude higher than their usual content. Thus, the presence of adenine nucleotides and ΔpH across the mitochondrial inner membrane are a necessary condition for PP_i inflow. It is worth noting that PP_i transport in plant chloroplasts probably follows an analogous mechanism [57,58].

In the case of an adenine nucleotide translocator described by Klingenberg and Vignais [59–63], there is an electrogenic $\Delta\psi$-dependent exchange of external ADP^{3+} for internal ATP^{4-}. In energized mitochondria, K_m for ATP is significantly higher than it is for ADP, and this results in a predominant transport of ADP into mitochondria and ATP release into the cytoplasm. Affinity for the translocator is significantly lower in the case PP_i. According to the data of D'Sousa and Vilson [30], for the translocator of rat liver mitochondria this value is equal to 1.25 mM. Affinity for ADP is known to depend little on mitochondrial energization. But for ATP, K_m shows a dramatic increase under energized conditions [63]. For PP_i, K_m does not change with the addition of an uncoupler, even though the rate of transport increases several-fold [30]. It is conceivable that $\Delta\psi$ across the membrane hinders PP_i from entering mitochondria and, like ATP it carries a more negative charge than does ADP. Let us note that both in coupled and in uncoupled mitochondria K_m for PP_i is higher than that for ATP (1–5 μM in uncoupled and up to 0.2 mM in coupled mitochondria), not to mention ADP (1–5 μM in coupled and uncoupled mitochondria). At the same time, the PP_i concentration in the cytosol apparently does not exceed several micromoles, while inside mitochondria it may attain as high as 1–20 mM. The possibility is not excluded, therefore that under physiological conditions mitochondrial PP_i may be transported to the cytosol in exchange for ADP in coupled mitochondria, and probably for ATP in uncoupled ones, and may thus increase the content of adenine nucleotides in mitochondria. Apparently, it is due to this process that accumulation of adenine nucleotides in mitochondria under some hormonal effects takes place [64]. But the reverse movement of these metabolites is also possible. For example, in uncoupled mitochondria the rate of PP_i transport increases, and it may operate as an energy source for sustaining the transmembrane potential. Significantly, the value of a potential generated at the expense of PP_i is the higher, the lower is its initial level across the membrane [35].

III. Control of PP_i metabolism in mitochondria

At first when, coupled to oxidative phosphorylation, PP_i biosynthesis was discovered, we thought that the PP_i function in mitochondria in general would be similar to that of ATP. But experimental data of the last 10 years have demonstrated that the regulatory function of PP_i in mitochondria is probably the most important. Meantime, the regulation of PP_i accumulation and utilization in mitochondria is not yet clear. The problem is complicated by the presence of the soluble PPase, in addition to the coupling enzyme, in the matrix [24,45,47]. The formation of a soluble PPase from the membrane one under the storage of the isolated enzymes and the identity of catalytic subunits demonstrates that these are related enzymes [48]. Moreover, the membrane coupling PPase is not tightly bound to the membrane. As a result we frequently obtain mitochondrial preparations that are not only incapable of oxidative phosphorylation coupled PP_i biosynthesis but, in the absence of PPase inhibitors, immediately hydrolyze the PP_i formed during activation of butyrate through ATP. Possibly, during in vivo functioning of mitochondria the amount of soluble and membrane-bound enzymes may change. And the possibility is not excluded that mitochondrial swelling or contraction has also some part to play.

The PP_i accumulated in mitochondria either as a result of oxidative phosphorylation or butyrate activation was quickly hydrolyzed with the addition of uncouplers.

10 years ago, V. Skulachev [65] postulated that PP_i in the cell may operate as a buffer for the electrochemical H^+ potential. Indeed, having succeeded in effecting PP_i transport via the translocator of adenine nucleotides, we could observe a PP_i-dependent generation of $\Delta\psi$ across the mitochondrial membrane [35] and PP_i-dependent synthesis of ATP from ADP [23]. Reverse electron transport at the expense of PP_i bond energy was earlier demonstrated in Baltscheffsky's laboratory [20].

It was found during investigations of ATP and PP_i accumulations in mitochondria that the maximum of these synthetase activities correspond to different conditions and that factors, stimulating one process, suppress

the other. Thus, it was observed that both processes are regulated by the respiratory rate differently and exhibit a different dependence on low concentrations of dinitrophenol [66].

Earlier it was shown for chromatophores of photosynthesizing bacteria that biosyntheses of ATP and PP_i proceed at different values of an incubation medium redox potential created by the pairs ascorbate – phenasinemetasulfate or ascorbate-dichlorophenolindophenol [67,68]. Experiments on changes of the redox potentials of respiratory chain carriers in rat liver mitochondria revealed the same regularity for ATP and PP_i syntheses [66]. In contrast to ATP biosynthesis, the biosynthesis of PP_i proceeded under a higher reduction of respiratory chain components. More active hydrolysis of PP_i by PPase corresponded to the conditions of maximum of ATP synthesis, while ATPase activity corresponded to those of PP_i biosynthesis.

There is evidence that a more fluid membrane is needed for maximum ATP synthesis than for PP_i synthesis [69].

The recent results [70–72] seem to indicate that the major role of tightly bound ATP and ADP on F_1 and CF_1 ATPases consists in the modification of their activity. There are a few reports in the literature also concerned with the binding of PP_i to the mitochondrial and chloroplast coupling factors [72–77]. It was found that nucleotides and PP_i competed for binding to the chloroplast and mitochondrial F_1. PP_i, added to the incubation medium, decreases the rate of inactivation caused by ADP and also decreases the level of tightly bound ADP. According to Vignais and co-workers [73], beef heart mitochondrion F_1 has three PP_i-binding sites: one a high-affinity site ($K_d \simeq 1\ \mu M$), and two lower-affinity sites ($K_d \simeq 20\ \mu M$). These authors suggested that PP_i and ADP share the same binding sites on F_1 and that PP_i interacts with the same amino-acid residues. It was also found that the binding affinity of F_1 for P_i was increased by a concentration of PP_i lower than 100 μM and it was decreased by a higher concentration of PP_i. This biphasic effect of PP_i on P_i binding was not observed with ADP, which, at all the concentrations tested, inhibited P_i binding. For all the other effects, PP_i mimicked ADP. The absence of any effect of PP_i on ATPase activity might be explained by its rapid release from F_1 at the beginning of catalysis [73]. It is not clear whether a possible binding of PP_i on mitochondrial F_1-ATPase has a role to play for its activity regulation in vivo.

Concluding this section, I would like to say that, despite the clarification of the important role of PP_i for mitochondrial functioning and, despite the achievements scored in the study of certain reactions involving PP_i, very little is known about the regulation of these processes and their connection with the essential functions of mitochondria.

IV. Mitochondrial pyrophosphatases: PP_i-hydrolyzing and synthesizing enzymes

Although the equilibrium of the reaction $PP_i \rightleftharpoons 2\ P$ lies far to the right, under certain positions it is possible to observe PP_i synthesis by isolated pyrophosphatase. The synthetase function of the enzyme at first was observed in 1958 [78,79]. Later it was proved that, like F_1-ATPase, which synthesizes the tightly bound ATP, inorganic pyrophosphatase from yeast synthesizes PP_i, also tightly bound to the enzyme [80–84]. Avaeva and co-workers [84] suceeded in obtaining preparations that contained up to two molecules of PP_i per molecule of yeast pyrophosphatase, or one PP_i molecule per enzyme subunit. In the opinion of Boyer and co-workers [80], it is not the synthesis but the low rate of release of the synthesized product from the enzyme active site that is the limiting step for PP_i accumulation in the solution. As a consequence, under equilibrium conditions the amount of PP_i bound to the enzyme may exceed by several orders of magnitude its content in the aqueous phase.

Among all known solvents, water has the most cooperative structure. This structure is destroyed in the presence of such compounds as dimethyl sulfoxide, ethylene glycol, poly(ethylene glycol) and others [85–88]. De Meis and co-workers [89–91] showed that, with an increase in the concentration of these organic solvents in solution, free energy of pyrophosphate bond hydrolysis ($\Delta G°$) and the observed constant of equilibrium between phosphate and pyrophosphate are decreased. Thus, with the increase in the content of poly(ethylene glycol) (with a molecular mass of 8000 Da) to 50% (w/v) in a solution with 0.9 mM $MgCl_2$ and 1 mM phosphate at pH 8.0, $\Delta G°$ falls off from -3.5 to 1.3 kcal/mol, and the observed equilibrium constant from 346 to 0.1 M. The authors believe that $\Delta G°$ of phosphate-containing compounds depends on the difference in the solvation energies of substances entering into a reaction and those formed as a result. According to these authors [92], in the presence of organic solvents the K_m of PPase for P_i is significantly lower and the level of PP_i synthesis is higher.

In the view of Behrens and De Meis [91], the active site of the coupling membrane pyrophosphatase is located on the border between the hydrophobic and the hydrophilic regions of the membrane. When $\Delta\tilde{\mu}_{H^+}$ is generated across the membrane, the active site of the enzymes shifts several nanometers into the hydrophobic region. The PP_i thus formed transfers from the hydrophobic to the hydrophilic region until an equilibrium is attained. With the disappearance of the potential, the active site relocates to the hydrophilic environment again and hydrolysis of the synthesized product sets in. Such translocations were earlier suggested for Ca^{2+}-ATPase of the sarcoplasmic reticulum [93].

The success in the study of mitochondrial pyrophosphatases is due largely to the improved techniques of their separation and purification [24,25,47,48,94]. Two forms of the enzyme were found to be present in mitochondria: the membrane pyrophosphatase, not tightly bound to the interior side of the mitochondrial inner membrane, and the soluble one, located in the mitochondrial matrix [45,47,49,52,95,96].

Membrane pyrophosphatase from beef heart mitochondria has a molecular mass of about 185 kDa and contains four types of subunit: α (28 kDa), β (30 kDa), γ (40 kDa) and δ (60 kDa). The putative structure is $\alpha\beta\gamma_2\delta$. In the case of the soluble pyrophosphatase with a molecular mass of 60 kDa, two subunits, identical to the catalytic α and β of the membrane enzyme, were isolated. The putative structure is $\alpha\beta$. Present in rat liver mitochondria are also the soluble and membrane pyrophosphatases [45,48,49,96]. Extraction with cholate produces the soluble pyrophosphatase of the matrix (I) and two membrane forms with molecular masses 120 kDa (II) and 210 kDa (III). The authors [49] suggest that pyrophosphatase II, which comprises the two different subunits, α (28 kDa) and β (35 kDa), is part of a more involved structure, complex III.

The presence of two enzymatic forms of PPase was likewise shown for yeast mitochondria [97].

The soluble and the membrane forms of pyrophosphatases from beef heart mitochondria revealed a striking similarity in their properties [24,45,47,50,94]: high lability, identical isoelectric points and specificity, pH optima at 7–9 and analogous activation by Mg^{2+} and Co^{2+}. An analysis of hydrolysis kinetics for both enzymes showed that the complex Mg^{2+}-PP_i serves a substrate for them and that the binding of two Mg^{2+} ions per active site of the enzyme is an essential condition of activity. Both enzymes are inhibited by fluorine ions [23,45,55,95,96,98], by Ca^{2+} [48] and less by P_i and ATP [48]. They are rapidly inactivated in the absence of SH reagents [24,25,47,94]. SH groups, though not within the active site, exert a significant effect on the activity of the enzymes. A study of the reactivity of SH groups from the membrane PPase of submitochondrial particles led to the conclusion that part of the surface of its catalytic subunits is within the membrane [99]. The possibility is not to be excluded that the level of reduction of pyridine coenzymes and SH groups of glutathione and mitochondrial proteins affects the reduction level of SH groups of mitochondrial pyrophosphatases, thus regulating enzyme functioning in vivo.

The study of mitochondrial pyrophosphatases also showed them to be similar in many respects to yeast pyrophosphatase [100]. In both cases a complex, containing Mg^{2+} ions and pyrophosphate, is reactive: three Mg^{2+} ions and pyrophosphate per subunit in yeast pyrophosphatase, and the same amount per molecule of the enzyme in mitochondrial pyrophosphatases. In either case the enzymes have, apart from the active sites those capable of binding inorganic phosphate. Both the yeast and the mitochondrial pyrophosphatases are inhibited by fluoride. And finally, both enzyme types, in the presence of phosphate, are capable of in vitro synthesis of pyrophosphate in the enzyme activity site [101]. Significantly, in the membrane enzyme the maximal incorporation in to PP_i was achieved at a lower concentration of phosphate than in the case of the soluble membrane. It is proposed that herein lies the effect of non-catalytic subunits of the membrane pyrophosphatase.

Isolation and purification of the coupling membrane pyrophosphatase from photosynthesing bacteria [102–106] posed even greater difficulties. This enzyme proved to be tightly bound to the membrane and highly labile upon its extraction by cholate or Triton X-100 in the presence of high concentration of Mg^{2+} and ethylene glycol. The activity of the isolated enzyme was stimulated by the addition of phospholipids, of which cardiolipin was found to be most active. In contrast to cholate, when enzymatic activity is wholly dependent on the presence of a phospholipids, Triton X-100 reconstitutes enzymatic activity to 50%.

Compared with the coupling pyrophosphatase of photosynthesizing bacteria, the mitochondrial coupling pyrophosphatase is not tightly bound to the membrane and is washed off easily during ultrasonic treatment of mitochondria or with 0.25 M sucrose in the case of submitochondrial particles [51]. A membrane pyrophosphatase from beef heart mitochondria, isolated according to the method described in Ref. 24 or 94, was found to contain a phospholipid with the predominance of phosphatidylcholine [107]. The soluble form of the enzyme contained no phospholipid, but was easily lipidized in the presence of a total mitochondrial phospholipid, phosphatidylethanolamine or various phosphatidylcholines [69,107–109]. This was accompanied by some stabilization of the enzyme and by stimulation of its activity measured at 30 °C.

In our study [69,107–110] on the effect of temperature on the rate of PP_i hydrolysis by soluble and membrane pyrophosphatases of beaf heart mitochondria substantial differences in the behavior of these enzymes were detected [108–111]. For the soluble enzyme this dependence was linear in the Arrhenius plots, whereas the membrane enzyme displayed two inflections – at 18 °C and 26 °C. But after the lipidization of the soluble enzyme, Arrhenius plots exhibited inflections coinciding with the temperatures of the phase transitions of the phospholipids used. These data attested to the mitochondrial coupling pyrophosphatase activity being controlled by the physicochemical state of the surrounding phospholipids.

A comparative study of ATP and PP_i biosyntheses in mitochondria showed these two processes to be differ-

ently regulated by the viscosity of phospholipids from the mitochondrial inner membrane. In mitochondria with a more liquid membrane, the ATP biosynthesis proceeds more vigorously than does the PP_i synthesis, which increases with the increased viscosity of the membrane [69].

V. The possible role of PP_i in calcium and phosphate metabolism in mitochondria

We know from inorganic chemistry that inorganic polyphosphates – inorganic pyrophosphate and diphosphonates among them – taken at very low concentrations prevent crystallization of calcium carbonate and hydroxyapatite [112–118], probably as a result of adsorption on seed crystals, which prevents both the growth and dissolution of the crystals [118–120]. In addition, aggregation of individual crystals of hydroxyapatite is inhibited in the presence of PP_i [117,121]. In human urine and blood plasma, PP_i was identified as a major compound inhibiting phosphate and calcium precipitation from these liquids [2]. In cartilaginous tissue it was assigned a special role in the regulation of calcium/phosphate metabolism [122].

Only scant direct evidence is available on the role of this compound in calcium/phosphate metabolism in mitochondria. So let us try and assess what we know about the role of PP_i in cartilage calcification and some other processes.

It is known that energy-dependent transport of Ca^{2+} in mitochondria, accompanied by the simultaneous uptake of phosphate, results in the accumulation of hydroxyapatite-containing granules in the mitochondrial matrix [123–131]. Mitochondria, obtained by the water-free method, contain much more of such granules [130]; with the use of aqueous methods there is less calcium and phosphate in mitochondria, the Ca/P ratio is lower, and it varies strongly from experiment to experiment [129]. The amount of accumulated calcium depends on the presence of ADP (ATP) and Mg^{2+} in the medium, and may reach 3 μmol per mg of the protein. Dinitrophenol, added to such mitochondria, leads to the rapid disappearance of the granules, with the calcium being released. Three steps of energy-dependent deposition of calcium have been established [124,125]: the formation of small granules on the interior side of the mitochondrial inner membrane (mainly on the cristae), the appearance of small granules in the matrix and the growth of the granules to 500–1000 Å. Electron microscopy revealed fine granulation of larger granules – they were found to be absolutely amorphous and surrounded by a transparent envelope [125]. The literature data [132,133] give grounds for the assumption that the calcium-containing granules of mitochondria are surrounded by a phospholipid membrane that contains cardiolipin. This is highly consistent with the properties of acidic phospholipids which, in the presence of Ca^{2+} and phosphate, form a Ca-phospholipid-PO_4 complex, stimulating the precipitation of hydroxyapatite [134–137]. Also, it is well known that interaction of Ca^{2+} with cardiolipin causes the membrane to convert to a non-bilayer structure [138] and this may unstring liposome-like vesicles and induce them, together with phosphate and calcium, to transfer into the mitochondrial matrix.

The above-described granules contain calcium, phosphorus, magnesium and carbonate in the ratio 30 : 17 : 4 : 4 [139–141]. Not a unique phenomenon, they are similar in the structure to granules found in tapeworms [142–144] in *Tetrahymena pyriformis* [145–147] and in the matrix vesicles of calcifying cartilages [147–150]. *T. pyriformis* likewise have PP_i and pyrophosphatase in membrane-enveloped granules. It was demonstrated that Ca^{2+} accumulation in these granules depends on the presence of Mg^{2+} and P_i and is an energy-dependent process. The uncoupler, dinitrophenol, inhibits the phosphate transport and PP_i accumulation and as a consequence, the formation of granules.

Electron microscopy investigations of matrix vesicles of calcifiable cartilages and collagenic fibrills of bone tissue has revealed the presence of granules analogous to those found in the mitochondrial matrix [127,131]. It is suggested that, during calcification, the matrix vesicles are the locus of primary accumulation of the extracellular mineral phase, and their calcification is an obligatory step of subsequent calcification fibrils [149]. These vesicles, surrounded by a phospholipid membrane, were found to possess, apart from Ca, Mg and P_i [151–162] also PP_i [163,164] and a number of enzymatic activities: those of alkaline phosphatase, pyrophosphatase, Ca^{2+}-ATPase and nucleoside triphosphate : pyrophosphohydrolase [148,151,152,165–170]. The former three activities are probably caused by the same enzyme. The PP_i content in the calcifiable tissue progressively increases from the quiescence to the calcification zone. Yet the PP_i/P_i ratio is higher in the quiescence zone and drops significantly in the process of calcification. The role of PP_i here is believed to consist in accelerating the precipitation of amorphous calcium phosphate, in the stabilization of the amorphous state of this precipitate and in its slow conversion to hydroxyapatite [122]. The stabilization of the amorphous state of calcium phosphate and hydroxyapatite involves, besides PP_i, also Mg^{2+} and some proteins [122,171–174].

But the latter steps of mineralization are inhibited by PP_i [122]. The PP_i level in these tissues must be attained as a result of the coordinated and subtly regulated work of nucleosidetriphosphate : pyrophosphohydrolase and

pyrophosphatase. The phosphatase activity of the enzyme is probably not realized, and it performs the function of a phosphate transporter across the vesicular membrane [175].

It is possible that Ca^{2+}-ATPase discharges the function of a Ca^{2+} transport.

The question about the presence of PP_i and pyrophosphatase in calcium-phosphate granules of the mitochondrial matrix is still open. But such a possibility is not excluded – first, because present in calcium-loaded mitochondria is PP_i [40], and second, as a result of affinity of PPases for hydroxyapatite.

It was demonstrated in experiments on Ca^{2+} transport in mitochondria that this process is stimulated in the presence of phosphate and ATP [125–126], with both the latter compounds being transported into mitochondria simultaneously with Ca^{2+} ions. PP_i, added to mitochondria, causes a rapid release of Ca^{2+} into the medium [55].

The possibility is not ruled out that under these conditions other ions, e.g., Mg^{2+}, may also be released. Vercesi and Lehninger [55] believe that PP_i, though it enters mitochondria, is not hydrolyzed, for the mitochondria are incubated in a 2 mM phosphate-containing medium. However, mitochondrial pyrophosphatases are weakly inhibited by phosphate [47]. Also, there is evidence that in the presence of diphosphonates – pyrophosphatase inhibitors – Ca^{2+} release from mitochondria is likewise inhibited [176,177]. In addition to this, Ca^{2+} ions also inhibit mitochondrial pyrophosphatases. Thus, the question of PP_i hydrolysis in the process of Ca^{2+} release from mitochondria is yet to be elucidated. It will be noted that phosphate, too, may be a factor causing the same effect [178]. At a PP_i concentration about 20 μM, i.e., close to the real intracellular concentration [10,42], the rate of Ca^{2+} release after a 2 min lag period was about 2 nmol/mg of protein per min for rat heart mitochondria. The Ca^{2+} efflux is not accompanied by a decrease of the membrane potential or by a change of the ADP/O ratio. The addition of Mg^{2+} ions inhibits the process significantly, probably due to the fact that Mg-PP_i is not transported by the translocator.

It is known that the content of Ca^{2+} ions in mitochondria depends on the degree of reduction of pyridine coenzymes, particularly, NADPH [179–183]. Accordingly, PP_i is a more effective stimulator of Ca^{2+} release from mitochondria in a pyruvate- and malate-containing medium than in that containing succinate in the presence of rotenone.

Now, are mitochondria involved in calcification? There is no final answer to this question as yet. Many authors [126–128,131,184–186] maintain that mitochondria may be implicated in extracellular calcification by accumulating calcium in the form of granules and then releasing it, i.e., in a sense they serve as a calcium and phosphate depot in the tissue. Yet it is also possible that mineralization of mitochondria is related to their own metabolism but not to cartilaginous calcification.

Comparing cartilaginous tissue calcification processes with the accumulation of hydroxyapatite-containing granules in mitochondria, we find many common features. It may be suggested that in the case of mitochondria PP_i likewise has a very significant part to play for sustaining the amorphous state of hydroxyapatite granules and, in high concentrations, for dissolving hydroxyapatite and for transferring the accumulated calcium to the cytoplasm. Thus, even from the available evidence, we may conclude that inorganic pyrophosphate may play a very important role in cellular metabolism with respect to the level of calcium and possibly, of adenine nucleotides in mitochondria and cytosol.

VI. Conclusions

One of the most significant and best understood functions of PP_i in a cell is that of the regulation of the intensity of biosynthetic processes. Indeed, hydrolysis of this compound by PPases pulls the equilibrium of the reactions toward synthesis.

Yet we may conceive of such a coupling between two processes when the product, formed as a result of the former is consumed by the latter. One such case is when RNA biosynthesis in the nucleus is coupled to polymerization of the released PP_i and to formation of high-polymer polyphosphates, probably implicated in the regulation of chromatin activity [187].

On the other hand, the accumulated PP_i may be transported into another cellular compartment. A graphic case in point is furnished by fluxes of PP_i and adenine nucleotides across the inner mitochondrial membrane, e.g., during butyrate activation. The available experimental evidence invites the suggestion that in a living cell PP_i may be involved not only in the regulation of adenine nucleotide levels in mitochondria and in the cytosol, but also in the redistribution of Ca^{2+} and K^+ and, possibly, Mg^{2+}, between these compartments.

It was noted that PP_i, added or formed as a results of short-chain fatty acid activation in mitochondria, imitates the action of some hormones, specifically glucagon [11,188]. It is known that here, in parallel with cAMP, PP_i is formed from ATP as well. For that matter it is quite possible that PP_i could be responsible for the change of some mitochondrial functions under glucagon action. All the more so in the light of fresh data that PP_i, in the presence of K^+ ions, is capable of causing mitochondrial swelling and thus of altering their functional state [64,188].

The significant role of PP_i in the regulation of bone tissue calcification and the possible role of mito-

chondria in this process in the capacity of a calcium depot was discussed above.

PP_i, the ancient high-energy compound in evolutionary terms, has preserved to this day some functions of inorganic polyphosphates in higher eukaryotes [52]. Thus, PP_i-dependent phosphorylation of membrane proteins was detected in liver cells, and G-6-*P* synthesis by G-6-*P*ase in microsomes [5–7]. In the living cell PP_i is also employed as a transporter of a number of cations. Thus, among tested compounds like ATP, ADP, GTP, CTP and PP_i, the latter proved to be the most effective for mobilizing iron from transferrin and their transport into mitochondria [189]. The uptake process represents a complex series of reactions: mobilization of iron from transferrin with formation of ferric-iron-pyrophosphate, reduction and sequestering of ferric-iron-pyrophosphate and binding of ferrous iron to ligands of the mitochondria.

The possibility is not ruled out that the coupling membrane PPase of mitochondria may be a relic of the once widespead pyrophosphate energetics of the ancient forms of life. In *R. rubrum*, in the opinion of Baltscheffsky et al. [190], the main function of membrane PPase is the energy-dependent biosynthesis of PP_i. In this sense nothing certain can be said about the coupling PPase of mitochondria. Here the situation must be more complex. In mitochondria, the enzyme is the target of various effects, the most significant among them being the transmembrane electrochemical H^+ potential, the level of the redox potential of respiratory carriers, the viscosity of the phospholipid component of the membrane, etc.

Coupling PPase is pulled either toward hydrolysis or toward synthesis, depending on the functional state of mitochondria, which is probably determined on the mitochondrial matrix volume. The mechanism of the regulation of this process needs further study.

Summing up, we should stress that PP_i performs a great variety of functions in cell metabolism. Further investigations of its role in energy and basal metabolism are of paramount significance for a correct understanding of the functioning of mitochondria as well as the whole living cell.

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